CHARACTERIZING AND GENETICALLY MODIFYING OSCILLATORIA TO DEGRADE BPA

Presented by Grace Long

In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Environmental Science

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Dennis Mishler
Supervising Professor

Date

_____ _____

Norma Fowler
Honors Advisor in Environmental Science

Date

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Department: Environmental Science

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Characterizing and genetically modifying *Oscillatoria* to degrade BPA

Grace Long

ABSTRACT

Bisphenol A (BPA) is becoming an increasing environmental concern. It is a hormone disruptor, and its presence in natural waterways causes some aquatic species to experience problems in growth, reproduction, and development. An effective BPA removal system is needed. Genetically modifying a type of cyanobacteria—*Oscillatoria*—with a gene from a fungus—*Trametes versicolor*—may provide a solution. The *T. versicolor's* gene produces a laccase with the ability to degrade BPA in the presence of butylhydroxytoluene—a molecule naturally produced by *Oscillatoria*. Before transforming *Oscillatoria*, however, it is necessary to understand the genus. In this study, I characterized the growth of *Oscillatoria brevis*, *Oscillatoria lutea*, and *Oscillatoria prolifera* as well as their ability to naturally fluoresce. Additionally, I determined some of the species' antibiotic resistances: *O. brevis* was found to have some resistance to carbenicillin, gentamicin, and kanamycin; *O. lutea* was found to have some resistance to carbenicillin, gentamicin, and kanamycin; and *O. prolifera* was found to have some resistance to gentamicin, kanamycin, and spectinomycin. Furthermore, I attempted to conjugate the *Oscillatoriae* with a plasmid containing GFP and antibiotic resistance to carbenicillin, kanamycin, or spectinomycin. Because the cyanobacteria are not axenic (pure, without contaminants), different organisms in the microbial community were unintentionally transformed instead. Finally, I also created genetic parts to be used in Golden Gate Assembly once the conjugation procedure is successful.

INTRODUCTION

I set out to create and implement an effective BPA removal system due to the increasing threat that it poses to the environment. Because it is a hormone disruptor, some aquatic species' growth, reproduction, and development can be negatively impacted after exposure to BPA (*Bisphenol A Action Plan*, 2010). Additionally, some governments have taken action to phase out the manufacture of BPA because of public concern over its impact on human health, though these governments (the US and the EU) have found no evidence to back up public concerns (*Bisphenol A Action Plan*, 2010; Guerra, Kim, Teslic, Alaee, & Smyth, 2015). On the other hand, the Canadian government has banned the import, advertisement, and sale of items with BPA due to its own conclusion that the molecule could be harmful to humans at current levels (Guerra et al., 2015). Although the effect of BPA on human health is still debated, the molecule certainly poses a significant problem as a pollutant in the environment.

Although wastewater treatment plants are currently able to remove most BPA from water, some BPA does get released back into the environment, contributing to the overall influx of BPA. It has been shown that biological aerated filter processes are most effective in BPA removal with a median value of 95% efficiency in wastewater treatment plants (Guerra et al., 2015). Biological aerated filter processes employ

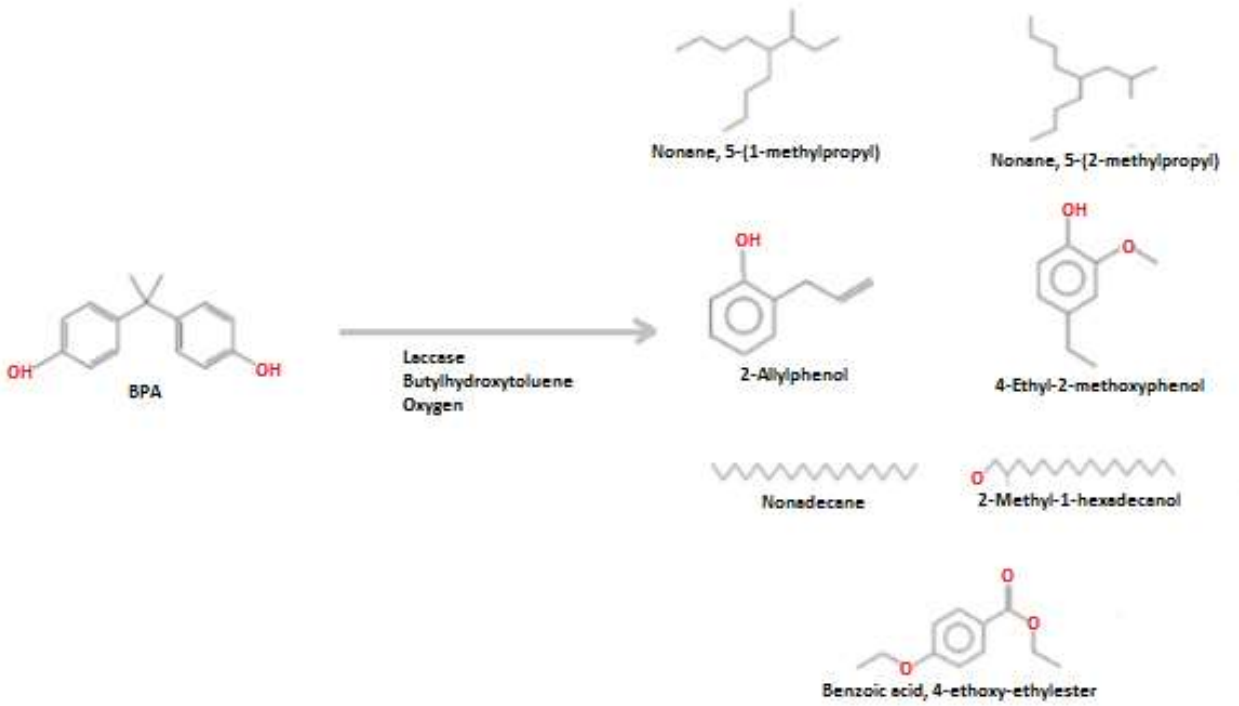


Figure 1: BPA degradation via the system used by Trametes versicolor

a bioreactor tightly packed with media to which organisms attach and grow (*TECHNOLOGY DETAIL FACT SHEET : Biological Aerated Filter (BAF)*, n.d.). This would indicate that naturally occurring BPA degradation processes are not only a viable solution to BPA pollution, but a preferable and effective one.

Trametes versicolor, a species of fungus, naturally has the ability to degrade BPA because of its ability to produce a certain laccase—a type of multicopper oxidase thought to be nearly ubiquitous in fungi (Solomon, Sundaram, & Machonkin, 1996). To degrade BPA, the *T. versicolor* laccase requires the presence of butylhydroxytoluene (known as a mediator molecule; Erkurt, 2015) and oxygen (Kang & Kondo, 2002). This process is illustrated in **Error! Reference source not found.**. *Oscillatoria*, a genus of cyanobacteria, naturally produces both of these molecules (Babu & Wu, 2008). By inserting the *T. versicolor* laccase gene into *Oscillatoria*, I hope to create an effective BPA degradation system. The *Oscillatoria*'s ability to produce butylhydroxytoluene and to photosynthesize (thereby producing oxygen) would provide the necessary molecules for the laccase gene to degrade BPA.

To develop this BPA degradation system, the organisms being worked with first have to be understood. *Oscillatoria* is not commonly worked with, nor had it been used in Professor Jeff Barrick's lab previously. Thus, *Oscillatoria brevis*, *Oscillatoria lutea*, and *Oscillatoria prolifera* were all studied to determine their natural antibiotic resistances. I additionally attempted to conjugate them using green fluorescent protein.

Although *Oscillatoria* are not commonly studied in synthetic biology, cyanobacteria as a whole have been used in the field, so some broad expectations on what to expect from the genus shaped my experimentation. Many cyanobacteria, including *Oscillatoria*, have very

long doubling times compared to the organisms typically used in molecular biology, making their genetic modification a relatively slow process. Additionally, cyanobacteria have several defense mechanisms in place to resist or prevent changes to their genome.

One such defense is the presence of endonucleases, which cleave foreign DNA that gets taken up by the cyanobacteria. Additionally many cyanobacteria produce an exopolysaccharide layer which can physically prevent foreign DNA from being taken up (Stucken, Koch, & Dagan, 2013). Also, in terms of *Oscillatoria* and other filamentous cyanobacteria, the successful conjugation of one cell does not necessarily mean the successful conjugation of neighboring cells.

Furthermore, I understood that the organisms I was working with would not be axenic (pure). The samples that I used for experimentation were each part of their own microbial community, meaning organisms besides *Oscillatoria* lived around and in concord with the cyanobacteria. Typically, genetic modification should be performed on an axenic culture. However, *Oscillatoria* are particularly difficult to purify. One reason for this is that the cyanobacteria and the other organisms in the community are all motile and consequently cannot be separated by moving one away from the rest—they move as a unit. Another factor is that the exopolysaccharide layer that *Oscillatoria* are coated in acts as a "slime tube," which essentially anchors other microbes to the cyanobacteria. Thus, the entire xenic samples were used during several parts of experimentation while I continuously attempted to purify the samples.

MATERIALS AND METHODS

I obtained *O. brevis*, *O. lutea*, and *O. prolifera* on BG-11 agar from the UTEX Culture Collection of Algae at the University of Texas at Austin and maintained them in liquid BG-11 or on BG-11 agar. I bought BG-11 from UTEX or made it following UTEX instructions (Peña, 2016). Additionally BG-11 agar was made following UTEX instructions (Peña, 2016). In this paper, "media" refers to BG-11 unless otherwise specified. Briefly, BG-11 contains 17.6 mM $NaNO_3$, 0.23 mM K_2HPO_4, 0.3 mM $MgSO_4$, 0.24 mM $CaCl_2$, 0.031 mM citric acid, 0.021 mM ferric ammonium citrate, 0.0027 mM Na_2EDTA, 0.19 mM Na_2CO_3, and 1mL/L BG-11 trace metals solution, all in deionized water. For solid media, agar and sodium thiosulfate pentahydrate are added. BG-11 trace metals solution contains 46 mM H_3BO_3, 9 mM $MnCl_2$, 0.77 mM $ZnSO_4$, 1.6 mM Na_2MoO_4, 0.3 mM $CuSO_4$, and 0.17 mM $Co(NO_3)_2$, all in deionized water.

GROWING CONDITION CHARACTERIZATION

I made several cultures of each species of *Oscillatoria* and placed them in eight different locations: on a bench under synthetic light at 20°C, on two different south facing windowsills at 26°C, on an east facing windowsill at 26°C, on a west facing windowsill at 26°C, in an incubator at 28.7°C (shaking at 150 rpm for two days, then not shaking), in an incubator at 30°C (shaking at 200 rpm), and in an incubator at 37°C (not shaking for two days, then shaking at 200 rpm). At least one culture of each species was placed in each location. I created each culture under sterile conditions, meaning the workspace was wiped down with ethanol before a Bunsen burner was lit in the center of the workspace. If open, the cultures were kept in

the vicinity of the flame (meaning the space in which the heat from the burner could be felt), and they were never held higher than the flame

To create one culture, 5 mL of BG-11 was placed in a culture tube. A loop tool was soaked in 70% ethanol for at least 5 seconds, then held in the flame of the Bunsen burner until the metal of the loop tool glowed red. This sterilized loop tool was then used to scrape some of the organism (~1-4 mm^3) from the appropriate UTEX sample. This piece of cyanobacteria was then transferred to the liquid BG-11 in the culture tube. The finished culture was then placed in one of the locations detailed above for at least 12 days. In practice, different cultures were made on different days and left for different lengths of time, but my results compare 12 day old cultures. Growth was estimated visually as no measurements were taken.

Growing Procedure

Liquid cultures were created as described in Growing Condition Characterization. Agar cultures were created following a similar procedure but with the following adjustments: 1) ~10 mL of BG-11 agar was poured into a small plate or ~25 mL into a large plate and allowed to dry, and 2) instead of being swirled into liquid, the cyanobacteria were gently dragged along the agar until they were successfully transferred from the tool to the surface of the agar. Unless otherwise specified, cultures were left to grow in south facing windows.

Antibiotic Resistance

Antibiotic resistances to carbenicillin, gentamicin, kanamycin, spectinomycin, and streptomycin were tested for each organism. The plasmids pBTK501, pBTK519, and pBTK520 contain genes with resistance to ampicillin, kanamycin, and spectinomycin, respectively (depicted in Figure 2, Figure 3 and Figure 4). In Dr. Barrick's lab, stock solutions of these antibiotics are kept in the following concentrations: 100 mg/mL of carbenicillin, 5 mg/mL of gentamicin, 50 mg/mL of kanamycin, 60 mg/mL of spectinomycin, and 100 mg/mL of streptomycin. Each plate of BG-11 was made following UTEX instructions (Peña, 2016), with the modification of mixing in the appropriate amount of antibiotic (explained below) before pouring the plates.

In total, six plates were made to test each antibiotic. For each antibiotic, the concentrations (by volume) of antibiotic stock solution to media + agar were 0:1, 1:1000, 1:2000, 1:4000, 1:8000, and 1:16000. For example, a large plate would consist of 25 mL of media + agar, so 25 µL of antibiotic would be mixed in to obtain a concentration of 1:1000. Three lines were drawn on each plate to "divide" the plate into thirds. In each third was either *O. brevis*, *O. lutea*, or *O. prolifera* such that each species was on every plate. All plates were then left to grow in an east facing window (because of limited space in south facing windows).

Auto Fluorescence

1 mL of PBS was added to a microcentrifuge tube, then a loop tool was sterilized (using the same method described in Growing Condition Characterization). The loop was used to sterilely scrape some (~1-4 mm^3) of one organism from solid culture to be transferred to the PBS in the microcentrifuge tube. The piece was broken up and mixed through repeated pipetting using a micropipet. A small amount of the PBS/cyanobacteria mixture was applied to a microscope slide and observed using a fluorescence microscope. This procedure was followed for each *Oscillatoria* species.

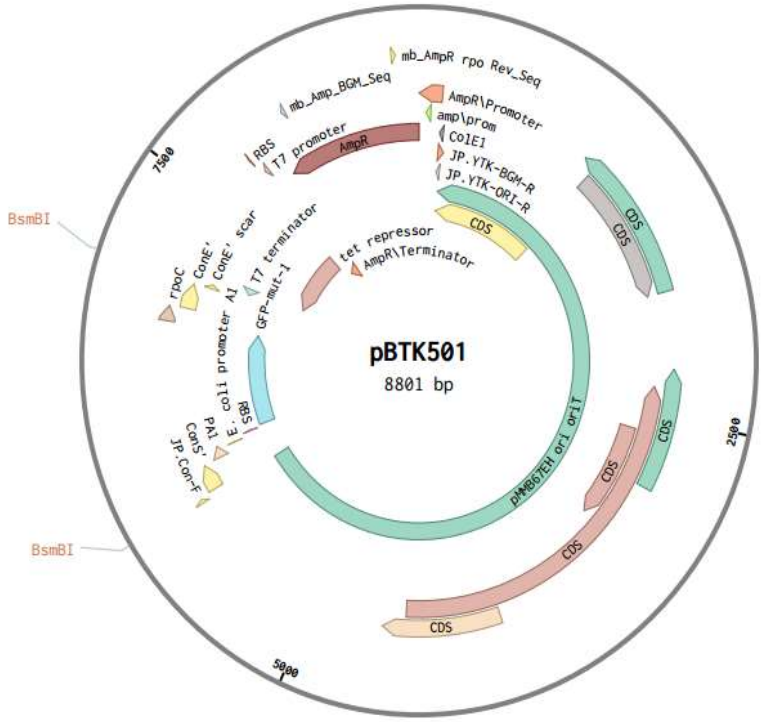


Figure 2: pBTK501, the plasmid containing ampicillin resistance. This figure was created by Jiri Perutka and obtained for this report from Benchling.

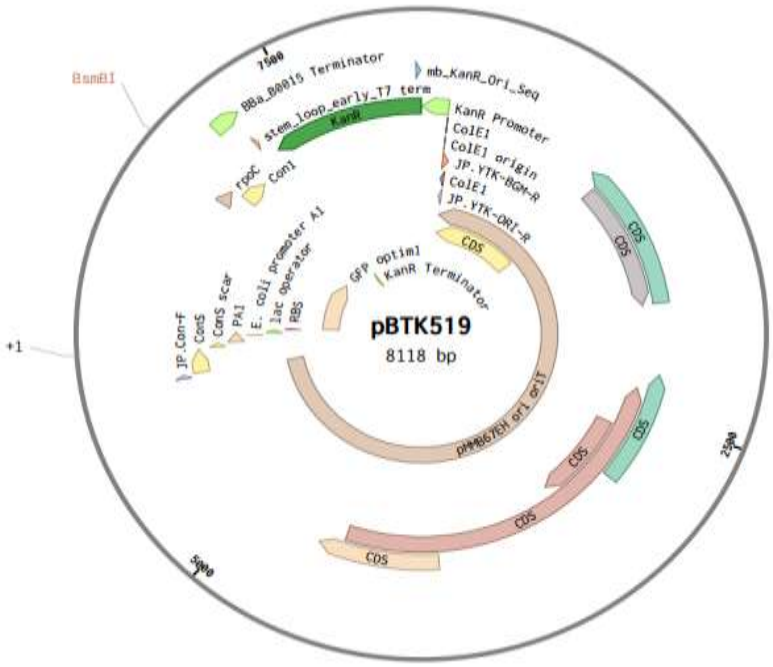


Figure 3: pBTK519, the plasmid containing kanamycin resistance. This figure was created by Jiri Perutka and obtained for this report from Benchling.

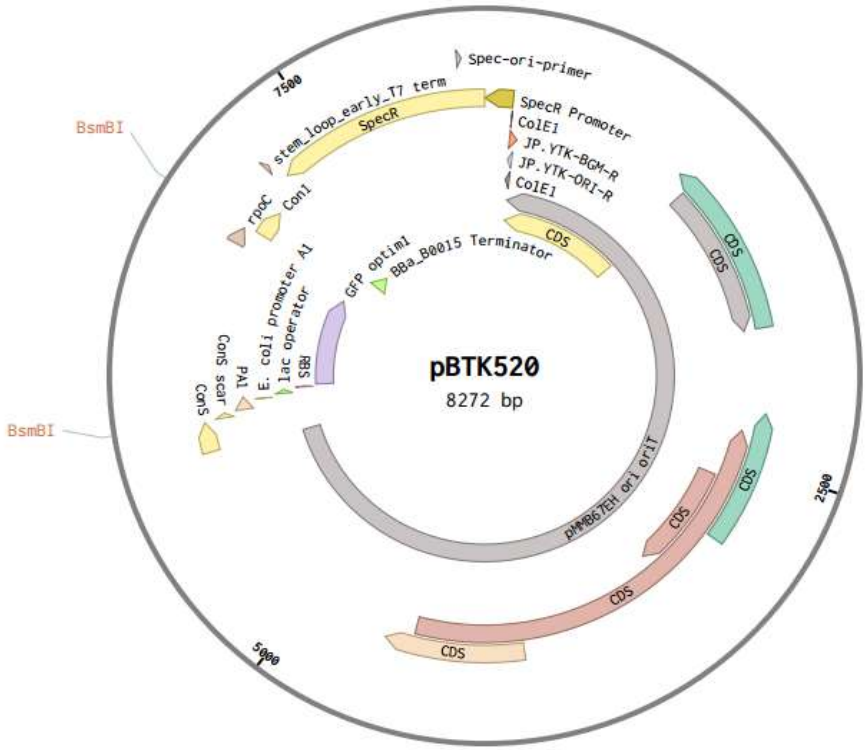


Figure 4: pBTK520, the plasmid containing spectinomycin resistance. This figure was created by Jiri Perutka and obtained for this report from Benchling.

Golden Gate Part Creation

The components necessary for entry vector creation for *Oscillatoria* are: an appropriate plasmid, a cyanobacterial promoter region (including an RBS), and the gene of interest—in this case, the *T. versicolor* laccase gene. Three gBlocks were created in Benchling and ordered from IDT: two promoter parts and one part containing the laccase gene. The promoter parts included cyanobacteria-appropriate promoters flanked by the sequence GCATCGTCTCATCGGTCTCAAATG before and the sequence TATGTGAGACCTGAGACGGCAT after (Barrick & Geng, 2016). The laccase part included the gene, codon optimized for cyanobacteria, followed by a 6xHIS tag and flanked by the sequence GCATCGTCTCATCGGTCTCAT before and the sequence GGATCCTGAGACCTGAGACGGCAT after (Barrick & Geng, 2016).

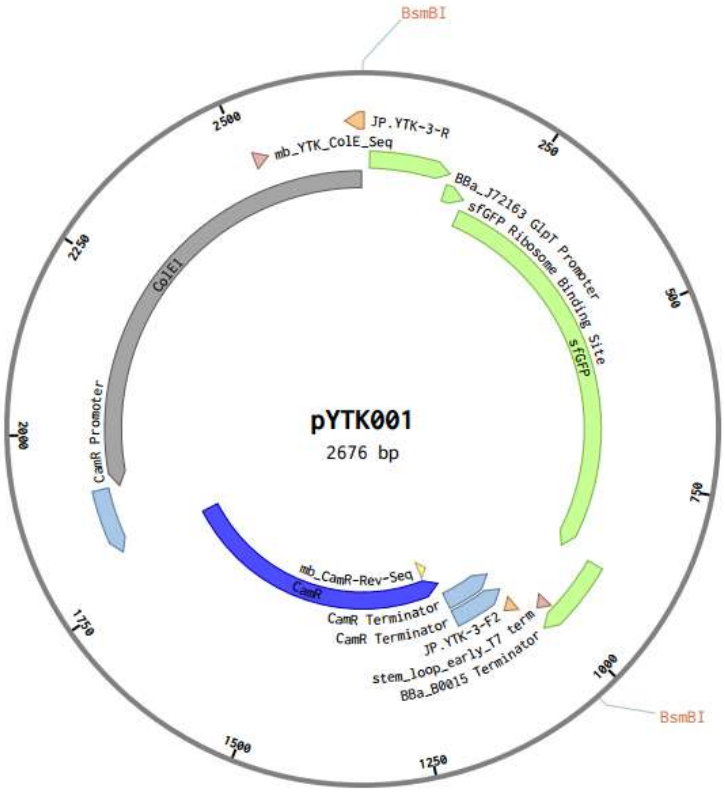


Figure 5: The plasmid used as the backbone for part creation. This figure was created by Jiri Perutka and obtained for this report from Benchling.

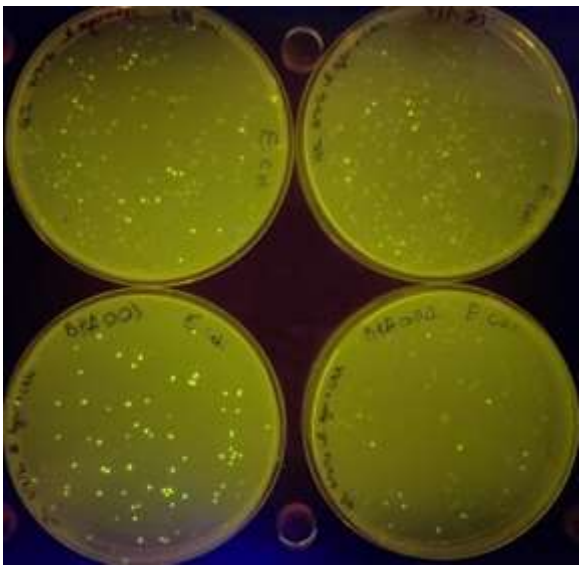


Figure 6: Golden Gate Part Creation

Once the gBlocks arrived from IDT, they were used to create parts for Golden Gate assembly. For each gBlock, the following were mixed (via pipetting) in a PCR tube: 1 µL BsmBI, 20 fmol pYTK_001 (the plasmid used, shown in Figure 5), 40 fmol of the gBlock, 2 µL 3.1 buffer, 2 µL 10 mM ATP, 1 µL 100 mM Dithiothreitol (DTT), and enough deionized (DI) water to bring the mixture to 20 µL. The mixture was incubated at 42°C for 30 minutes before adding 1 µL of T7 ligase. The mixture was incubated at 16°C for 30 minutes. The mixture then cycled through 5 minutes at 42°C and 5 minutes at 16°C 20 times. The mixture was then held at 55°C for 10 minutes and 80°C for 10 minutes. After that, the mixture could be held at 12°C indefinitely. The purpose of this procedure was to obtain the pYTK_001 plasmid with the desired gene in place of a green fluorescence gene.

To determine the success of this procedure, 2 µL of the mixtures were electroporated into TOP10 *E. coli* which were then allowed to recover in liquid LB with a 20 µg/mL concentration of chloramphenicol (Barrick & Geng, 2016). When streaked out on LB Agar with the same chloramphenicol concentration, the non-fluorescent *E. coli* colonies were suggestive of successful part creation, so they were grown up and made into stocks for Professor Barrick's lab. Figure 6 shows what a plate with fluorescent and non-fluorescent colonies might look like.

CONJUGATION

Conjugation of the cyanobacteria was attempted with a DAP auxotrophic *E. coli* containing the donor strains pBTK501, pBTK519, or pBTK520 (described in the Antibiotic Resistance section as well as Figure 2, Figure 3, and Figure 4). The basic method followed to conjugate the cyanobacteria was to 1) scrape up enough cyanobacteria to form a piece ~1-4 millimeters cubed, 2) place it into 5 milliliters of 0.5 M NaCl + media, 3) shake the culture tube at 150 rpm at room temperature (~20°C) for 1 hour, 4) place that cyanobacteria onto a plate with media that supports growth for the cyanobacteria and the donor strain (in this case, the media was BG-11 with DAP), 5) apply 25 µL of the donor strain in the same place as/on top of the cyanobacteria, and 6) after 24 hours, move the cyanobacteria to a plate with selective media (usually BG-11 with antibiotics and no DAP).

CONTAMINANT ISOLATION AND IDENTIFICATION

Contaminants were isolated using a loop tool to scrape across the "successfully conjugated" cyanobacteria cultures without scraping hard enough to collect any of the cyanobacteria itself. The loop tool was then streaked across BG-11 KAN plates to grow them up (the contaminants had been transformed with kanamycin resistance). They were then transferred to 10 different types of plates that could provide them with a carbon source: HS, HS + KAN, YPD, YPD + KAN, LB, LB + KAN, BG-11 + casamino acids + glucose, BG-11 + casamino acids + glucose + KAN, M9 + KAN, M9 + 2X KAN. The growth from both M9 plates was then used for the identification process.

To identify the contaminants, their genomic DNA was first extracted using an Invitrogen PureLink DNA Mini Kit. Within this kit, the protocol called Preparing Lysates—Mini Kit: Gram Negative Bacterial Cell Lysate was observed, followed by the protocols called Purification Procedure Using Spin Columns: Binding DNA, Washing DNA, Eluting DNA, and Storing DNA. Once the DNA was extracted, a Qubit was used to determine its concentration. PCR was then performed on the samples, followed by PCR purification. The Qubit was used on these new samples, after which they were prepared for sequencing at UT's Sequencing Facility. This preparation consisted of creating 12 µL samples that included 1 µL of primer and a concentration of 0.5 ng/µL of DNA. These samples were then sequenced by the Facility.

RESULTS

GROWING CONDITIONS CHARACTERIZATION

Part of characterizing the growth conditions of the *Oscillatoria* involved determining their natural antibiotic resistances. Figure 7 through Figure 14 show the growth that each culture experienced over a twelve day period from June 2, 2017 to June 14, 2017. The cultures in the windows showed significantly

more growth than those under synthetic light or in the incubators. In fact, those in the incubators showed very stunted growth or even died, in the case of the 37°C incubator (Figure 14). The cultures in both south facing windows (Figure 8 and Figure 9) showed the most growth out of all the cultures. These results are unsurprising as cyanobacteria naturally grow in sunlight. Furthermore, south facing windows are known to receive the most sunlight (compared to windows facing other directions) in the northern hemisphere. Additionally, the growing patterns of each of the species are shown below in Figure 15, Figure 16, and Figure 17. I used these results to understand what conditions were most ideal for these species to grow.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 7: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept on a bench under synthetic light at 20°C. These pictures were taken by Grace Long and Baltazar Zuniga.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 8: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in south facing windowsill A at 26°C. These pictures were taken by Grace Long and Baltazar Zuniga.

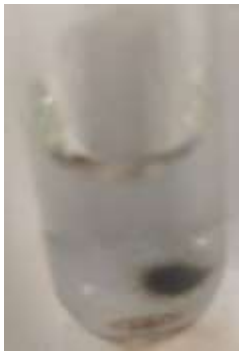
	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 9: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in south facing windowsill B at 26°C. These pictures were taken by Grace Long and Baltazar Zuniga.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 10: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in an east facing windowsill at 26°C. These pictures were taken by Grace Long and Baltazar Zuniga.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 11: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in a west facing windowsill at 26°C. These pictures were taken by Grace Long and Baltazar Zuniga.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 12: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in an incubator at 28.7°C (shaking at 150 rpm for two days, then not shaking). These pictures were taken by Grace Long and Baltazar Zuniga.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 13: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in an incubator at 30°C and shaking at 200 rpm. These pictures were taken by Grace Long and Baltazar Zuniga.

	Day 0	Day 12
O. brevis		
O. lutea		
O. prolifera		

Figure 14: Comparison of cultures on June 2, 2016 and June 14, 2016 (after 12 days of growth) when kept in an incubator at 37°C (not shaking for two days, then shaking at 200 rpm). These pictures were taken by Grace Long and Baltazar Zuniga.

Figure 15: O. brevis *growth pattern after 8 days of growth*

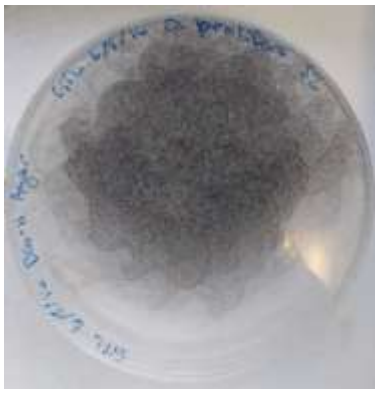


Figure 16: O. prolifera *growth pattern after 8 days of growth*

Figure 17: O. lutea growth pattern after 8 days of growth

ANTIBIOTIC RESISTANCE

The natural antibiotic resistances of the *Oscillatoria* is important in the creation of entry vectors for the organisms. If one of the cyanobacteria is already naturally resistant to one of the antibiotics used, transforming that cyanobacteria to possess that resistance would not make sense and would not be useful. Furthermore, the resistances of the *Oscillatoria* may be useful in ridding the cultures of contamination; it may be the case that the cyanobacteria have resistances to one or more antibiotics that the contaminants do not possess. *O. brevis* and *O. lutea* were both resistant to carbenicillin up to a concentration of 100 µg/mL, but both also showed signs of distress at a dilution of 6.25 µg/mL compared to their growth on non-antibiotic plates. *O. prolifera* showed no resistance to carbenicillin at any concentration. The growth of the strains on carbenicillin is shown in Figure 18. *O. brevis* and *O. lutea* were both unaffected by gentamicin, and *O. prolifera* only began to show signs of distress at a dilution of 5 µg/mL. The growth of the strains on gentamicin is shown in Figure 19. *O. brevis* and *O. lutea* were both resistant to kanamycin up to a dilution of 50 µg/mL, and *O. prolifera* was resistant up to a dilution of 25 µg/mL. However, all three began showing signs of distress at a dilution of 3.125 µg/mL. The growth of the strains on kanamycin is shown in Figure 20. All three were resistant to spectinomycin at all concentrations, but all three were extremely distressed at a dilution as low as 3.75 µg/mL. The growth of the strains on spectinomycin is shown in Figure 21. There was some unknown issue with the streptomycin plates used for the experiment that inhibited all growth for all three organisms. I do not believe that the lack of growth is due to the antibiotic, so these results are not shown.

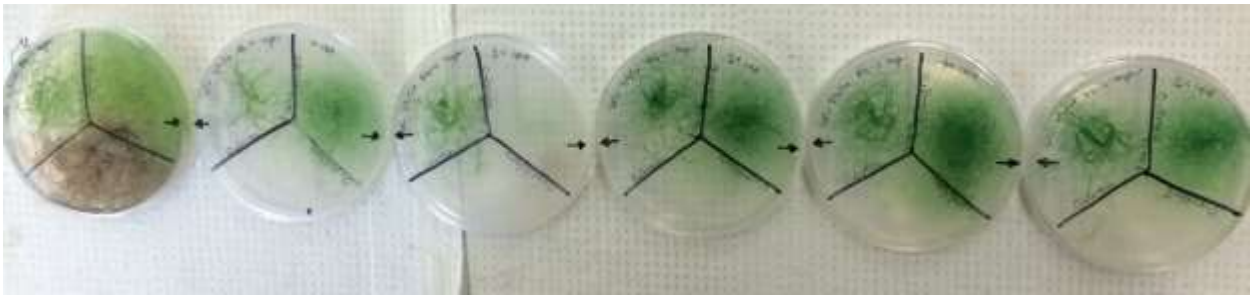


Figure 18: Growth of the Oscillatoria *strains on different concentrations of carbenicillin. On each plate,* O. lutea *is plated in the top left,* O. brevis *in the top right, and* O. prolifera *in the bottom. From left to right, the concentration of carbenicillin to media is 0 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL. This image was taken on October 11, 2016 after 15 days of growth. These plates were started by Grace Long. This picture was taken by Kimberly Corona.*

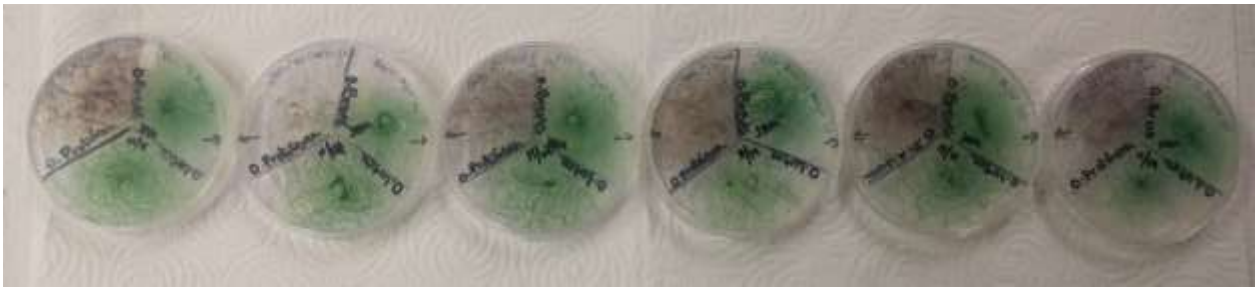


Figure 19: Growth of the Oscillatoria strains on different concentrations of gentamicin. On each plate, O. prolifera is plated in the top left, O. brevis in the top right, and O. lutea in the bottom. From left to right, the concentration of gentamicin to media is 0 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, and 0.3125 µg/mL. This image was taken on December 5, 2016 after 21 days of growth. These plates were started by Jennifer Martin. This picture was taken by Grace Long.

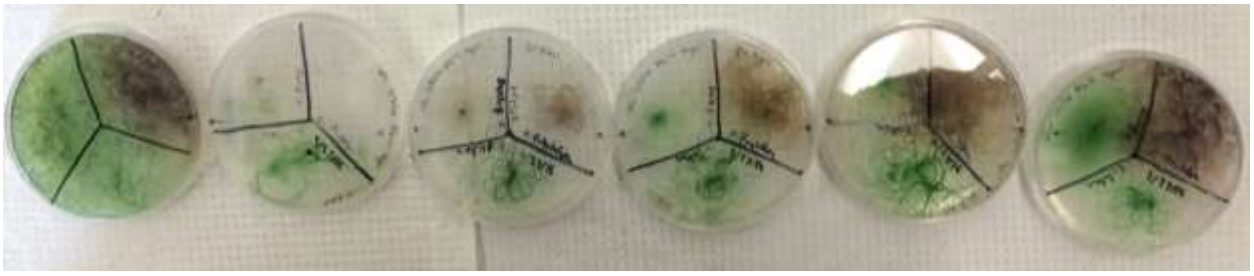


Figure 20: Growth of the Oscillatoria strains on different concentrations of kanamycin. On each plate, O. prolifera is plated in the top right, O. brevis in the top left, and O. lutea in the bottom. From left to right, the concentration of kanamycin to media is 0 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, and 3.125 µg/mL. This image was taken on October 13, 2016 after 16 days of growth. These plates were started by Daniel Hrncir. This picture was taken by Kimberly Corona.

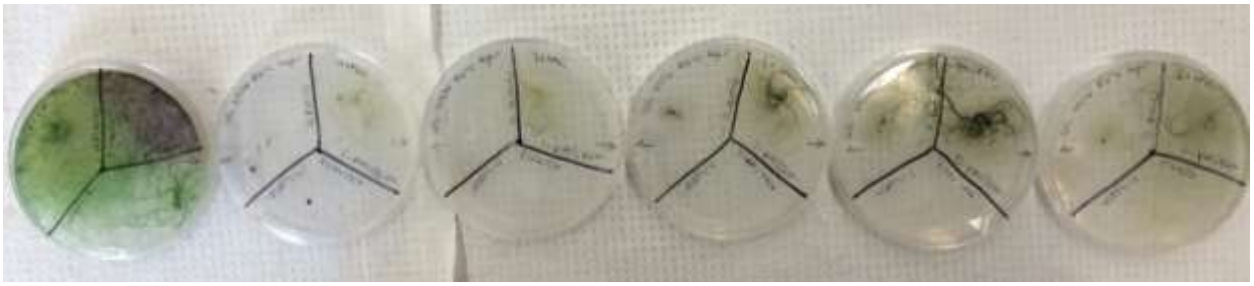


Figure 21: Growth of the Oscillatoria strains on different concentrations of spectinomycin. On each plate, O. prolifera is plated in the top right, O. brevis in the top left, and O. lutea in the bottom. From left to right, the concentration of spectinomycin to media is 0 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 7.5 µg/mL, and 3.75 µg/mL. This image was taken on October 13, 2016 after 17 days of growth. These plates were started by Vrinda Rajkumar. This picture was taken by Kimberly Corona.

CONJUGATION

As a proof of concept, I attempted to insert plasmids containing GFP and antibiotic resistances into the *Oscillatoria*—pBTK501 (ampicillin resistance, Figure 2), pBTK519 (kanamycin resistance, Figure 3), pBTK520 (spectinomycin resistance, Figure 4). My most successful conjugations occurred using pBTK519 (the plasmid containing kanamycin resistance, Figure 3). *O. brevis* was not noticeably transformed, however *O. lutea* and *O. prolifera* both seemed to have undergone successful conjugation before I examined them under a microscope (Figure 22). However, once observed under a microscope, it became clear that the cyanobacteria themselves were not fluorescing, but that other organisms in the microbial communities were. Figure 23 shows an example of the bacteria around the cyanobacterial filaments fluorescing.

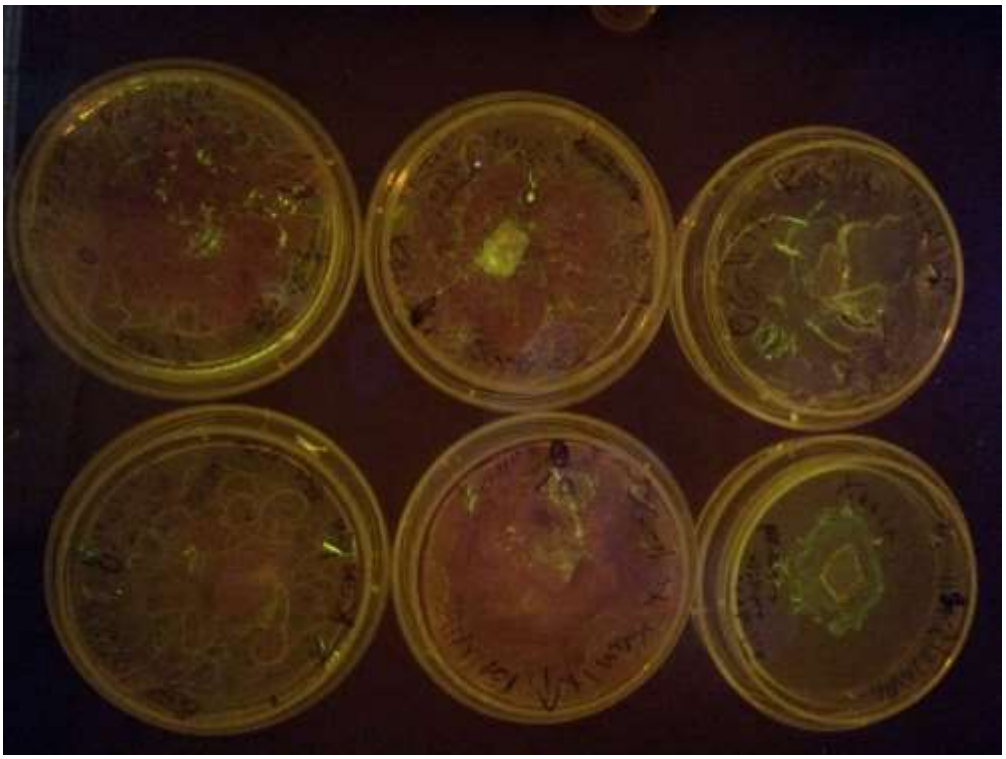


Figure 22: Results of conjugation with plasmids containing GFP and antibiotic resistance shown under blue light. The conjugations that showed the most promise were O. prolifera *and* O. lutea *conjugated with pBTK519 (kanamycin resistance)*

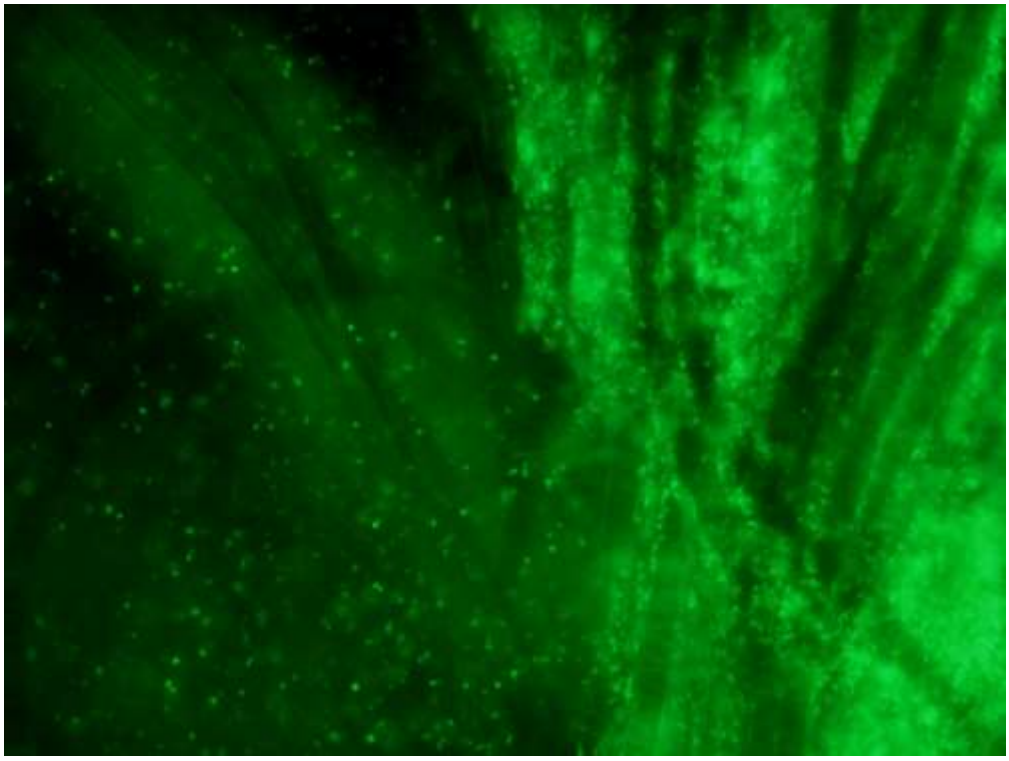


Figure 23: Successful conjugation of O. lutea *contaminants using a plasmid containing GFP and kanamycin resistance. The filamentous* O. lutea *is clearly non-fluorescent while the single-celled contaminants are. This picture was taken by Dr. David Nobles.*

CONTAMINANT IDENTIFICATION

Using RDP (which stands for The Ribosomal Databse Project ; "RDP," 2014), I was able to identify the *O. lutea* contaminants sequences that were returned from the UT Sequencing Facility as belonging the genus *Ochrobactrum* and likely the species *intermedium*. The alignment of DNA from *Ochrobactrum intermedium* with the DNA isolated from the contaminants is shown below in Figure 24.

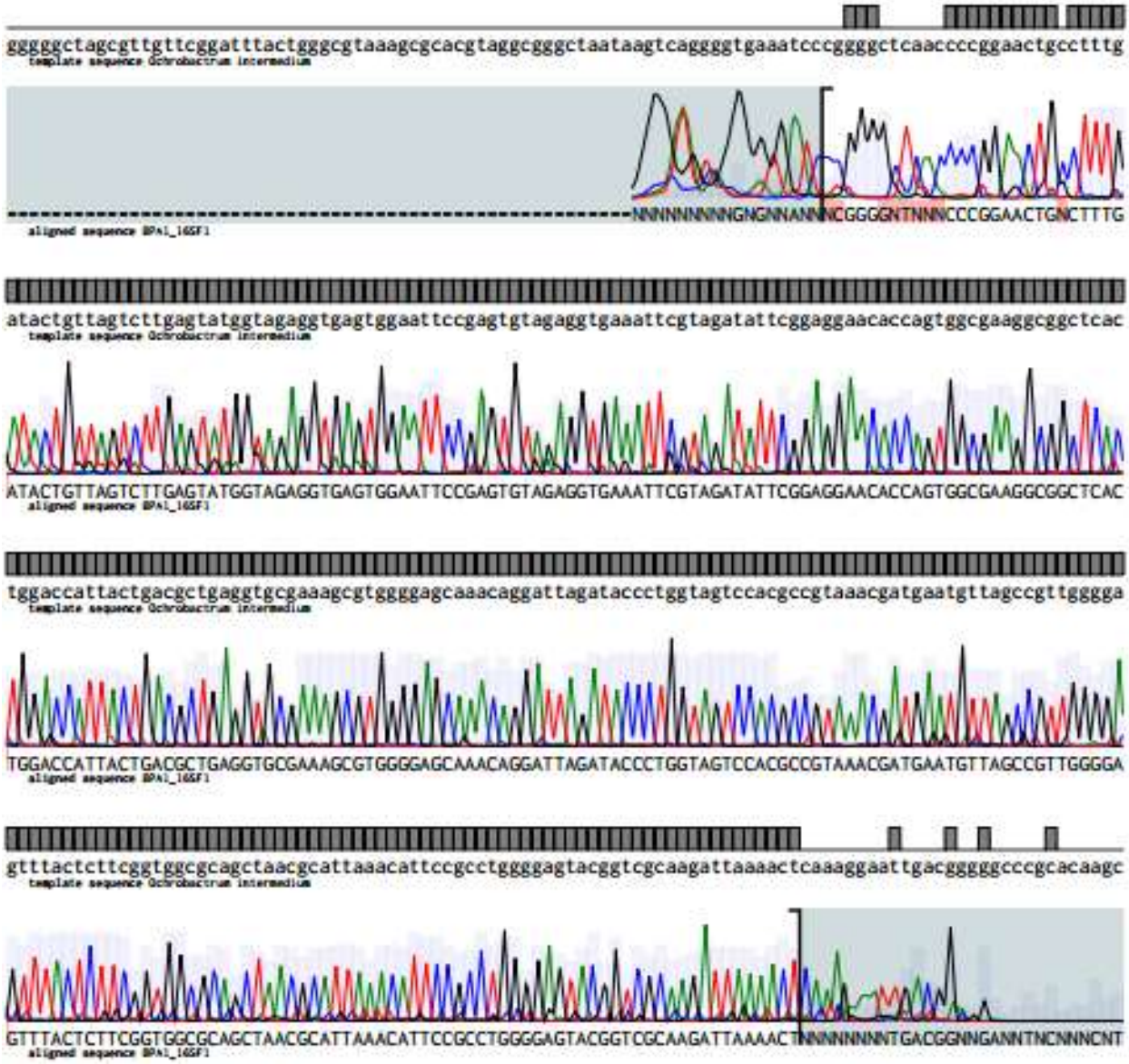


Figure 24: An alignment created in Benchling between Ochrobactrum intermedium *(top sequence) and the DNA isolated from the* O. lutea *contaminants (bottom sequence). The chromatogram was provided by the UT Sequencing Facility.*

DISCUSSION AND CONCLUSIONS

OSCILLATORIA CHARACTERIZATION

I spent several months learning about the basic behavior of *Oscillatoria brevis*, *Oscillatoria lutea*, and *Oscillatoria prolifera.* Learning the typical growth patterns that should be expected from each organism (Figure 15, Figure 16, Figure 17) as well as which environments are most suitable for the organisms—sunny windows—has made culture maintenance relatively manageable. Furthermore, I determined their resistance to several different antibiotics in order to have a fuller understanding of their behavior as well as to inform my actions when attempting the conjugation and axenification of the *Oscillatoria* (discussed more in the "Conjugation" and "Axenification" sections). *O. brevis* and *O. lutea* seem to be hardier than *O. prolifera* when taking only the antibiotics used in this study into consideration. Additionally, because the streptomycin results were inconclusive, this minimum inhibitory curve may need to be reassessed.

CONJUGATION

I attempted to conjugate the *Oscillatoria* cultures, which resulted in the successful conjugation of and identification of *Ochrobactrum intermedium* as opposed to any of the *Oscillatoria* themselves. Although I was attempting to conjugate the cyanobacteria itself, the fact that I successfully conjugated the *O. intermedium* is not necessarily a failure. It is possible that transforming the *O. intermedium* and reintroducing it into a microbial community with *O. lutea* could create an effective BPA degradation system. In such a system, the *O. intermedium* would provide the laccase while the *O. lutea* would provide the butylhydroxytoluene and oxygen. The three antibiotic resistances being considered for entry vector creation were ampicillin, kanamycin, and spectinomycin. The fact that all three species of *Oscillatoria* were resistant to spectinomycin at all concentrations would make spectinomycin a poor candidate for entry vector creation, meaning the plasmid pBTK520 is no longer a candidate in this respect. However, ampicillin/carbenicillin and kanamycin would still be viable antibiotic resistances to transform into the cyanobacteria using the appropriate concentrations.

OCHROBACTRUM INTERMEDIUM

Similar to *Oscillatoria*, *O. intermedium* is not commonly used in synthetic biology. Although I have successfully conjugated it, having a deeper understanding of its growth requirements will be necessary in creating an effective BPA degradation system. Thus, I will need to study it in a manner similar to how I studied the *Oscillatoria* strains. Subsequently, I will reattempt conjugation. Thus far, I have gathered that *Ochrobactrum* can be found in polluted soil and water, which lends credibility to its identification as a component of the microbial community with *O. lutea*. Furthermore, another strain of *Ochrobactrum* is unable to undergo to conjugation via individual mating but can do so as part of a microbial community (de la Cruz-Perera et al., 2013). This may be important in the attempted conjugation of *O. intermedium*.

AXENIFICATION

In the event that the "contaminants" prove not to create an effective BPA removal system, another option I will pursue will be to rid the *Oscillatoriae* of them, so that I can reattempt the conjugation of the cyanobacteria as originally planned. There are several different techniques that I can attempt to make the

cultures axenic, including: transforming the contaminants to self-destruct, using antibiotics to kill them, or separating the cyanobacteria and the "contaminants" through centrifugation. Because I have already successfully conjugated the bacteria coating the *Oscillatoriae*, I should be able to transform them with a "suicide gene" that would kill them post-conjugation. Additionally, because all three *Oscillatoria* have resistance to spectinomycin, it may be a good antibiotic to use in axenification; the *Oscillatoria* would presumably survive in spectinomycin while any contaminants would not. Likewise, gentamycin would also be especially useful for this purpose because all of the cyanobacteria were resistant at high concentrations. The other antibiotics tested for could also be used in axenification attempts at the right concentrations. I could also attempt to separate the components of the community through centrifugation. The different bacteria are different sizes and have different masses, so centrifugation should be able to stratify the larger, more massive cyanobacteria from the smaller, lighter "contaminants." Further work in this area may also reveal more methods of axenification that I can attempt.

BPA Degradation

Once *Oscillatoria* or *Ochrobactrum* has been successfully transformed with GFP as a proof of concept, I will then transform the appropriate organism with the laccase from *T. versicolor*. The organism will then be reintroduced into its microbial community and the effectiveness of the resulting system will be evaluated through the use of various assays. One assay will be to use HPLC to detect the presence of BPA in a solution before and after its introduction to the degradation system. I will also use a calorimetric assay to determine enzymatic activity (laccase production) in the transformed organism.

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